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Epistatic Interaction of LPL and PPARγ Genes in Adipocytes by Exposure to Dioxin

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ABSTRACT

Aim : To clarify the effects of dioxins on adipocytes during differentiation by analyzing gene expression of lipoprotein lipase (LPL) and peroxisome proliferator activated receptor (PPAR)γ.

Method : Murine 3T3-L1 preadipocytes were treated with 50 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and the messenger (m) RNA levels of PPARγ and LPL were measured during insulin-induced differentiation. To analyze epistatic interaction between PPARγ and LPL, we performed knock-down of PPARγ mRNA by small interfering (si) RNA and examined the relationship between the expression of PPARγ mRNA and LPL mRNA. We also examined the relationship between LPL and PPARγ when PPARγ was over-expressed in a stable transformant.

Results : Levels of LPL mRNA and PPARγ mRNA in TCDD-exposed cells were significantly lower than those in unexposed cells ($p=0.00053$ and 0.00078, respectively). The siRNA for PPARγ also reduced mRNA of PPARγ by 65.4% and that of LPL by 82.8%. However, when the PPAR gene was over-expressed, levels of LPL mRNA were higher than in untreated 3T3-L1 preadipocytes ($p=0.0005$), even after treatment with TCDD.

Discussion : The observed correlation between LPL mRNA and PPARγ mRNA suggests that LPL gene transcription is down-regulated, possibly through epistatic interaction with PPARγ, whose gene expression is primarily inhibited by TCDD. (Jikeikai Med J 2008 ; 55 : 19-24)

Key words : endocrine disruptors, adipocyte, peroxisome proliferator activated receptor γ, lipoprotein lipase, diabetes mellitus

INTRODUCTION

Of identified dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic to humans. TCDD has been shown to inhibit adipocyte differentiation[1]. In addition, several epidemiological studies have shown that dioxins can cause diabetes mellitus[2,3]. To cause this metabolic disturbance, dioxins interfere with the actions of insulin at insulin-sensitive organs, including adipose tissue, and eventually decrease glucose tolerance. Therefore, to understand environmental factors that might be associated with the increased worldwide prevalence of type 2 diabetes[4], we must clarify how dioxins affect metabolic pathways in adipocytes.

In adipocytes, peroxisome proliferator activated receptor (PPAR)γ and lipoprotein lipase (LPL) are both expressed abundantly[5]. In 3T3-L1 adipocytes, the LPL gene is up-regulated by PPARγ during insulin-induced adipocyte differentiation. LPL expres-

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sion is a marker of adipocyte differentiation, and PPARγ determines the adipocyte phenotype, including the stage of differentiation. Therefore, LPL is critical for the development of insulin resistance in adipose tissue when the function and expression of LPL are disturbed[6]. In the present study, we examined whether dioxin modifies adipocyte function, including the expression of LPL, an effecter molecule of insulin. Accordingly, we examined changes in LPL expression and analyzed the relationship between LPL and PPARγ after adipocytes were exposed to the dioxin TCDD. To investigate the mechanism of the observed change in LPL expression, we also analyzed the relationship between PPARγ messenger (m) RNA and LPL mRNA by knock-down through small interfering (si) RNA and by over-expression of PPARγ.

MATERIALS AND METHOD

1. Cell culture, induction of adipocyte differentiation, and TCDD treatment

Murine 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and were maintained in Dulbecco's modified Eagle's medium (Nikken Biomedical Laboratories, Fukuoka, Japan) with 10% calf serum at 37°C in a 5% CO_2 environment. To induce differentiation, 3T3-L1 cells at 2 days after the culture reached confluence were exposed to a differentiation medium containing 1 μM dexamethasone, 0.5 mM 3-isobutyl-1 methylxanthine, and 1 μg/ml insulin[7]. TCDD (50 nM) was then added to this culture medium for 7 days. The medium was changed every 2 days.

2. RNA extraction and slot blot analysis

Total cellular RNA was extracted from 3T3-L1 cells (RNeasy Mini kit, Qiagen Inc., Valencia, CA, U.S.A.). For slot blot analysis, 10 μg of the total cellular RNA was transferred onto a nylon membrane (Hybond N, Amersham Biosciences Corp., Piscataway, NJ, U.S.A.) by means of a Filtration manifold system (Life Technologies Inc., Grand Island, NY, U.S.A.) and were then subjected to hybridization to complementary (c) DNA murine PPARγ or LPL gene. The probes were labeled with the random primers method with α-^{32}P-dCTP (deoxycytidine triphophate) (Amersham Biosciences Corp.). Densitometric analysis of autoradiographs was performed with an image analyzer (BAS2000, Fujifilm Co., Tokyo, Japan) and analysis software (NIH Image V1.56, United States National Institutes of Health; available on the Internet at http://rsb.info.nih.gov/nih-image/). The level of the mRNA specific for each gene was standardized with the level of murine β-actin mRNA using the same blots.

3. Suppression LPL mRNA by siRNA

Duplex-DNAs siRNA targeted PPARs were purchased from Panomics (Redwood City, CA, U.S.A.). The siRNA duplexes were used for transfection with Lipofectamine™2000 (Invitrogen, Carlsbad, CA, U.S.A.) to 100-mm culture plates. The 3T3-L1 cells were treated to induce differentiation and culutured in 10 mL of Dulbecco's modified Eagle's medium with 10% calf serum without antibiotics. For each plate of cells, 4 μg of TranSilent siRNA PPAR Vector DNA and TranSilent siRNA Control Vector were transfected. The TranSient siRNA Vector DNA contains the following 3 DNA fragments (PPAR-F1: GCTGTCC TGGCTCAGATGGTTCAAGAGACCATCTAGCCAG GACAGCTTTTT; PPAR-F2: GGCCTCAGGCTAT CATTACTTCAAGAGAGTAATGATAGCCTGAGGC CTTTTT; and PPAR-F3: GCTGGTGTATGACA AGTGCTTCAAGAGAGCACTTGTCATACACCAGC TTTTT). The cells were incubated at 37°C in a CO_2 incubator for 2 days after transfection, and differentiation was induced. Seven days after differentiation had been induced, the cells were harvested and were subjected to extraction of total cellular RNA.

4. Over-expression of the PPARγ gene

To study 3T3-L1 cells that over-express PPARγ, stable transformants of 3T3-L1 cells were established with a plasmid cytomegalovirus vector containing the PPARγ gene. The stable transformants were then selected with geneticin for colony isolation and were designated as 3T3-L1/PPARγ cells. The 3T3-L1/PPARγ cells were exposed to the differentiation medium and TCDD. The first day of TCDD treat-

ment and differentiation-induction was defined as day 0.

The amount of LPL or PPARγ mRNA was determined with slot blot hybridization on the same day.

5. Statistical analysis

The level of each mRNA is presented as mean$\pm$SD relative abundance units (RAUs) with a maximum of 1.00 in unexposed 3T3-L1 cells standardized with each β-actin mRNA. The data were analyzed statistically with Student's t-test. Differences were considered significant when $p<0.05$.

Results

1. Suppression of LPL and PPARγ expression in TCDD-exposed 3T3-L1 adipocytes

To determine how LPL expression in adipocytes is affected by exposure to dioxins during the insulin-induced differentiation, we first examined levels of LPL mRNA after adipocyte differentiation was induced.

Levels of LPL mRNA in TCDD-exposed 3T3-L1 adipocytes were significantly lower than those in cells not exposed to TCDD (0.61 ± 0.05 RAU, $p=0.00053$; Fig. 1a and 2a).

Levels of PPARγ mRNA in TCDD-exposed adipocytes were significantly lower than those in unexposed cells (0.47 ± 0.01 RAU, $p=0.00078$; Fig. 1b and 2b). From these experiments, it appeared that PPARγ mRNA abundance was lowered by dioxin treatment, and that the low LPL by dioxin was accompanied at least by lower mRNA abundance of PPARγ.

2. Knock-down of PPARγ mRNA levels with a specific siRNA decreased LPL expression

To analyze the precise mechanism of the TCDD-induce decrease in LPL mRNA, we performed knock-down experiments; interfering with the PPARγ mRNA using a siRNA that was specific for PPAR instead of the TCDD exposure. Specific interference of PPARγ decreased levels of LPL mRNA (Fig. 2a and 2b). The PPAR siRNA reduced PPARγ mRNA to 65.4% (0.65 ± 0.11 RAU, $p=0.186$) and LPL mRNA to 82.8% (0.83 ± 0.38 RAU, $p=0.302$). Although the observed changes did not reach the level of statistical

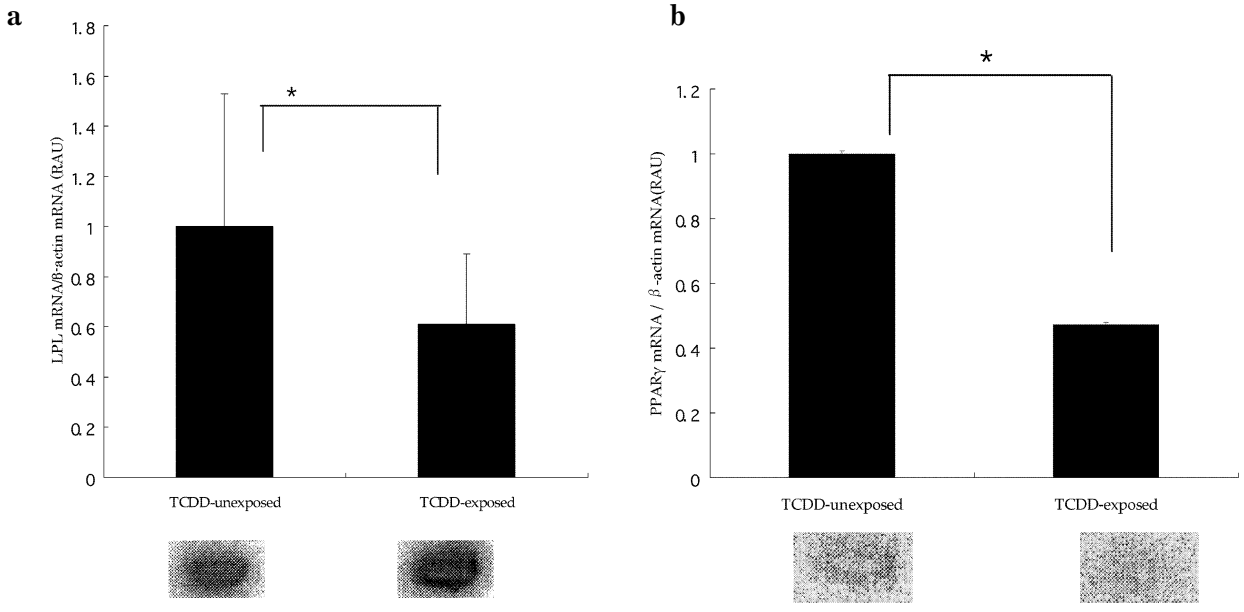

Fig. 1a, 1b. LPL and PPARγ mRNA expression in adipocytes exposed to TCDD
LPL mRNA (a) and PPARγ mRNA (b) in adipocytes were examined in adipocytes exposed to TCDD. Levels of mRNA were standardized with the level of murine β-actin mRNA. Levels of LPL and PPARγ mRNA in TCDD-exposed 3T3-L1 adipocytes were significantly lower than those in TCDD-unexposed cells ($p=0.00053$ and $p=0.00078$, respectively).

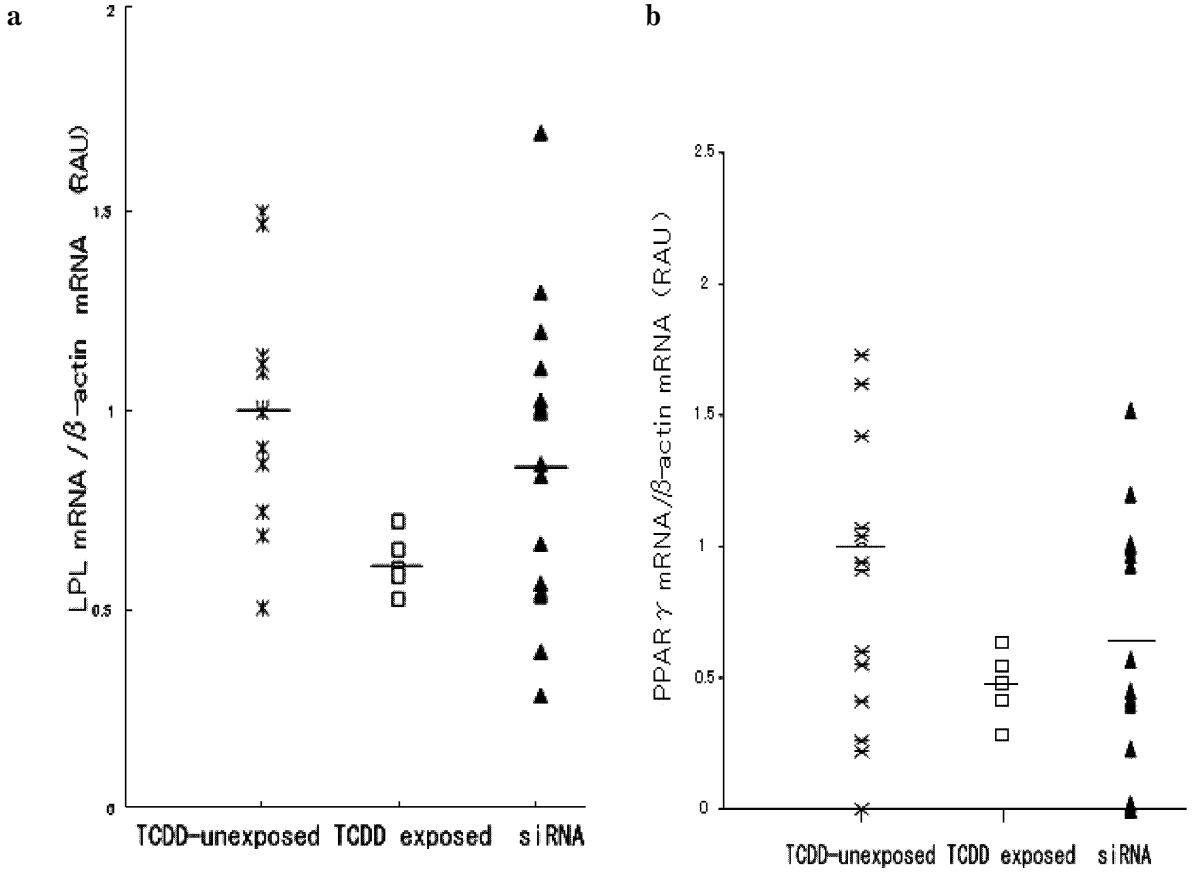

Fig. 2a, 2b. Correlation between LPL and PPARγ mRNA expression by knocked-down using PPARγ siRNA
Specific interference of PPARγ decreased PPARγ and LPL mRNA levels (65.4% and 82.8%, respectively). There was no significant difference in PPARγ and LPL mRNA levels between 3T3-L1 cells transfected by PPAR-siRNA or by a control vector. The horizontal lines indicate the mean of each group.

significance, down regulation of LPL expression resulted from the decrease in PPARγ without TCDD treatment.

3. *Restoration of LPL mRNA expression by over-expression of PPARγ in TCDD-exposed 3T3-L1 adipocytes*

To further investigate the relationship between PPARγ and LPL, we next observed correlation between mRNA abundance of PPARγ and LPL with TCDD exposure in PPARγ-over-expressed 3T3-L1 cells (3T3-L1/PPARγ). In this over-expression system, we confirmed the consistent over-expression of PPARγ mRNA. Even when the 3T3-L1/PPARγ cells were exposed to TCDD, levels of LPL mRNA and of PPARγ mRNA increased compared with those of TCDD-exposed cells (LPL RAU ; 1.29 ± 0.65,

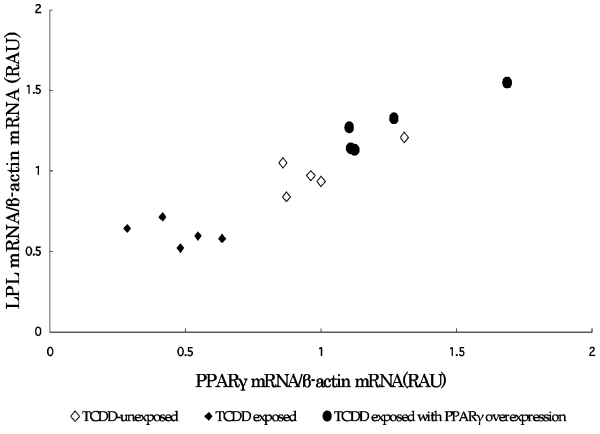

Fig. 3. Correlation between LPL mRNA and PPARγ mRNA expression by enhanced PPARγ gene expression
The relationship between PPARγ mRNA and LPL mRNA levels after TCDD exposure in PPARγ-over-expressing 3T3-L1 cells was examined. Levels of LPL mRNA and of PPARγ mRNA were correlated in each cell type ($p = 0.00004$, $r = 0.932$).

$p=0.0005$, PPARγ RAU ; 1.26 ± 0.17, $p=0.0005$, respectively). Furthermore, LPL mRNA levels were correlated with PPARγ mRNA levels in unexposed 3T3-L1 cells, TCDD-exposed 3T3-L1 cells, and TCDD-exposed 3T3-L1/PPARγ cells ($r=0.932$, $p=0.00004$; Fig. 3).

Discussion

Differentiating adipocytes are a major target of the dioxin TCDD, as observed in this study. Insulin stimulates expression of LPL at the transcriptional level in adipocytes and increases excretion of LPL from adipocytes. Extracellular LPL hydrolyzes triglycerides into free fatty acids and glycerol. The adjacent adipocytes then incorporate the increased fatty acids and accumulate triglycerides within adipocytes during differentiation[8-11]. Dioxins interfere with the actions of insulin adipocytes by inhibiting LPL mRNA expression and eventually inhibiting adipocyte differentiation. The expression of LPL is regulated by insulin and transactivation factors that affect adipocyte differentiation. In the present study we aimed to identify the mechanism by which dioxin antagonizes the actions of insulin, with a focus on LPL mRNA expression, as the expression of LPL is controlled at the transcriptional level. We found that exposure to the dioxin TCDD decreased LPL gene expression in differentiating adipocytes.

To determine the mechanisms for decreased expression of LPL by dioxins in differentiating adipocytes, it is important to analyze whether LPL expression is down-regulated by the attenuated activity of PPARγ because PPARγ is a potential transactivating factor for LPL transcription[7] and a key regulator of adipocyte functions, including glucose and fat metabolism. There are two possible mechanisms for PPARγ attenuation : the first is that the potency of the PPARγ molecule is decreased by dioxin exposure and that the potency of PPARγ regulates mainly the transactivation of the LPL gene ; the second possible mechanism is that the amount of PPARγ, including mRNA, is decreased rather than the potency of PPARγ and that the amount of PPARγ regulates mainly the transactivation of the LPL gene. In this study, we found that the latter

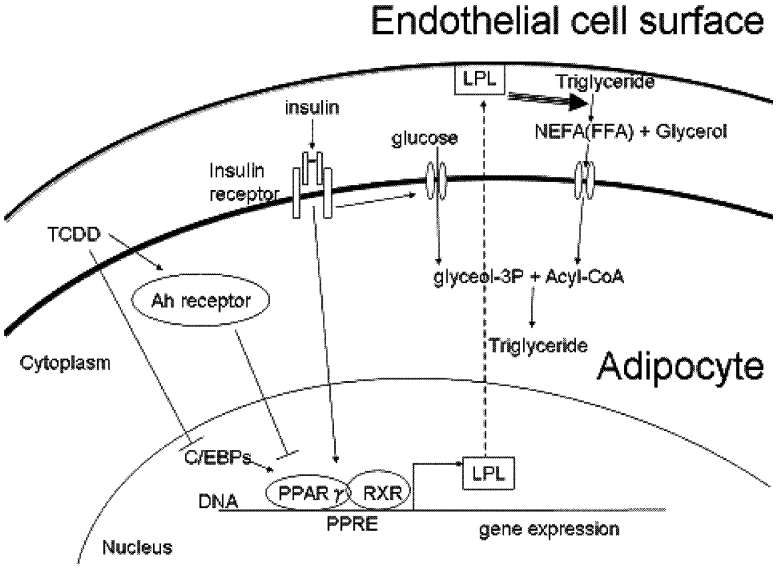

Fig. 4. The mechanism by which TCDD effects PPARγ and LPL in adipocytes
Two potential pathways for the attenuation of PPARγ gene transcription by exposure to dioxin. The dioxin-arylhydrocarbon receptor complex might inhibit PPARγ activity. Dioxin also might inhibit CCAAT/enhancer binding proteins to activate PPARγ.
Insulin increases expression of LPL in adipocytes. The expressed LPL is excreted from adipocytes and migrates to the endothelial cell surface and hydrolyzes triglycerides to provide fatty acids to adipocytes. However, insulin stimulates adipocytes to uptake fatty acids as well as glucose, activates hormone-sensitive lipase, and, eventually, promotes synthesis of triglycerides within adipocytes.

mechanism is most likely. In our experiment, LPL expression was restored even when the adipocytes were exposed to TCDD as long as the PPARγ gene was fully expressed by the cytomegalovirus promoter that would be never affected by the dioxin. Furthermore, we observed a strong correlation between the levels of LPL mRNA and PPARγ mRNA. These results could be explained by attenuated transcription of PPARγ by dioxin combined with arylhydrocarbon receptor or secondary inhibition through attenuation of CCAAT/enhancer binding proteins by dioxin[12–14]. Decreased transactivating potency of the PPARγ molecule is not likely because, in our experiment, once PPARγ levels were restored, LPL levels were also restored even after exposure to dioxin. The proposed mechanism of dioxin toxicity in terms of insulin action on adipocytes is summarized in Fig. 4[15].

Our present study suggests that endocrine disruptors, including dioxins, are potential environmental factors for increasingly prevalent disorders, especially insulin resistance syndrome, through effects on the differentiation and functions of adipocytes.

CONCLUSION

Exposure to TCDD resulted in inhibition of the insulin-induced differentiation of the 3T3-L1 preadipocytes with decreased expression of the LPL gene. The observed correlation between LPL and PPARγ mRNAs suggests that LPL gene transcription is down-regulated, possibly through epistatic interaction with PPARγ, whose gene expression is primarily inhibited by TCDD.

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